PATENT
Attorney Docket No.: A-59049/DJB

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Weiss *et al.*	Examiner: ZISKA, S.
Serial No. 08/149,508	Group Art Unit: 1804
Filed: November 9, 1993	
For: IN SITU MODIFICATION RESPONSIVE PROGENITOR CELLS WHICH CAN BE PROLIFERATED IN VITRO	

CERTIFICATE OF MAILING

I hereby certify that this correspondence, including listed enclosures, is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, DC 20231 on ~~30 April 1996~~ 7 May 1996 vh

Signed: Vicki L. Henry 7 May 96
Vicki L. Henry

DECLARATION UNDER 37 CFR 1.132

Commissioner of Patents
and Trademarks
Washington, DC 20231

Sir:

The undersigned, Brent A. Reynolds, hereby declares and states that:

1. I am a co-inventor of the subject application and I have read the arguments in the Official Action (mailed November 8, 1995).

2. The Examiner has rejected claims 1-7 and 23-31 stating that the specification is not enabling for the practice of the invention in humans and the claims must be limited to non-human mammals.

3. Under my supervision, a series of experiments was performed in the laboratories at Neurospheres Ltd., Calgary, Canada, to determine whether a population of relatively quiescent precursor cells, similar to the cells found in adult mice and described in the application, exists in the tissue lining the ventricles of primate forebrain. These experiments are described in paragraph 4A-4J below and confirm the presence of a similar precursor cell population in primates. A second series of experiments was performed to determine whether, as in mice, these precursor cells proliferate in response to growth factors administered *in situ*. These experiments are described in paragraphs 5A-5H below.

4. **Localization of Precursor Cells in the Primate CNS**

A. BrdU Injections: A male (3.5 year-old ~~juvenile~~ adult, weight 5.9 kg) Rhesus monkey (Macaca mulatta) was obtained from Health and Welfare Health Protection Branch, Canada (Ottawa) and kept on a standard laboratory diet with free access to water. In order to label the slow-cycling CNS precursor cells, the animal was injected intraperitoneally every eight hours for 5 days with a solution of bromodeozyuridine (BrdU) 2: 50/mL dissolved in 50% DMSO in 0.09% NaCl; 50 mg/kg; Sigma B-5002. Incorporated BrdU can be detected using immunocytochemistry.

B. Sacrifice: The animal was killed 2 hours after the last BrdU injection by an overdose of sodium pentobarbital. It was then perfused through the femoral artery with 2L of physiological saline containing 4000 - 5000U heparin for 10 - 20 min followed by 4L of Bouin's fix for 20 - 30 min at 4°C.

C. Tissue Preparation: The brain, spinal cord and a piece of gut (ileum) tissue were dissected out, cut into small blocks and kept in Bouin's post-fix solution at 4°C for up to 3 months. The tissue was then transferred in 0.1 M PBS + 0.02% Na-Azide, pH 7.4, at 4°C until ready for cryoprotection. The tissue was cryoprotected by serial incubations at 4°C in 10% sucrose, 20% sucrose and lastly in 20% sucrose and lastly in 20% sucrose: OCT (2:1). The tissue was then embedded in OCT and frozen in an isopentane bath at 45°C and stored at -70°C until sectioning.

D. Cryostat sectioning: Coronal sections of the rostral part of the lateral ventricle were cut at 30μm, from the frontal cortex to the appearance of the third ventricle, with a MICROM cryostat (-20°C) and each section was mounted on gel coated microscope slides and divided into six consecutive series. Slides were stored at -20°C until further processing.

E. BrdU immunocytochemistry: Slides were washed for 3 hr in PBS (Gibco BRL) to remove the Bouin's fixative and then incubated in 1 M HCl for 30 min at 60°C to denature and DNA. This was followed by 3 x 10 min washes in PBS. The sections were subsequently incubated overnight at room temperature in a primary antibody directed against single-stranded DNA contained BrdU (mouse monoclonal, 1:50; diluted in 0.1 M PBS containing 0.3% triton X-100 and 10% normal goat serum; Becton). After 3 x 10 min washes in PBS, the slides were incubated at room temperature for 60 min in goat anti-mouse CY3 (1:1000; Jackson). Following a final 3 x 10 min wash in PBS the slides were coverslipped under FluorSave (Calbiochem) and sealed using nail polish. BrdU immunoreactive cells were then examined using florescent microscope.

F. Quantification of proliferating cells: The total number of BrdU-labeled cells surrounding the lateral ventricle was counted using a 40 x

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objective and an ocular grid (250 x 250 μm) which was aligned along the wall of the lateral ventricle or along the contour of the caudate nucleus extending away from the lateral ventricle. The distribution of the cells was recorded by counting the number of cells within each successive ocular grid placement along the entire dorsal to ventral extent of the lateral ventricle and caudate nucleus. To establish the distribution of the BrdU-labeled cells in the rostro-caudal axis of the lateral ventricle, six sections were counted for each representative interaural point. Representative atlas co-ordinate interaural points were taken from the "Stereotaxic Atlas of the Monkey Brain, Macaca Mulatta" (Snider and Lee, University of Chicago Press, 1961) and from "A Stereotaxic Brain Atlas for Macaca Nemestrina" (Winters, Kado and Adey, University of California Press, 1969).

G. Labeled Cells of the Lateral Ventricle: We found a large population of proliferating precursor cells in the subventricular zone of the lateral ventricle in adult primate CNS. Attached Figure 1 represents the number of BrdU-labeled precursor cells found throughout the rostro-caudal axis for the lateral ventricle. As can be seen in Figure 1, the number of BrdU-labeled precursor cells in a 30μm section of the lateral ventricle ranges between 5 and 685 cells along the rostro-caudal axis. The peak number of BrdU-labeled precursor cells is found where the caudate nucleus juxtaposes the head of the nucleus accumbens (representative interaural line A20). At this level, most of the BrdU-labeled precursor cells (up to 54%) are found highly concentrated in the region which extends from the tip of the lateral ventricle at the junction between the nucleus accumbens and the caudate nucleus. The rest of the cells (about 36%) are scattered along the subventricular zone of the wall of the lateral ventricle, and about 10% are found in the region extending dorsally to follow the border of the caudate

nucleus until they reach the internal capsule. Rostral to the peak (interaural line A20), the number of BrdU-labeled precursor cells gradually decreases to about one third and falls repidly when the lateral ventricle is no longer in contact with the caudate nucleus (interaural line A25). Moving caudal from the peak, the number of BrdU-labeled cells decreases gradually at first, then quickly falls to about one quarter of this when the most caudal aspect of the nucleus accumbens is reached (interaural line A17). There is a further gradual decrease in the number of BrdU-labeled precursor cells found around the lateral ventricle as one moves caudal to interaural line A2. It is interesting to note that in the primate forebrain, as long as the lateral ventricle is juxtaposed to the caudate nucleus some BrdU-labeled precursor cells are found. In contrast, the portion of the lateral ventricle wall not in contact with the caudate nucleus has much fewer, if any, BrdU-labeled precursor cells. So the location of the proliferative precursor cells in adult primate is closely associated with the caudate nucleus. Furthermore, the distribution of the cells along the subventricular zone of the wall of the lateral ventricle is not a continuous string of cells; rather, cells are distributed in clusters (data not shown). Each cluster has an average of about 30 cells and can be found up to 200 μm away from the wall of the ventricle but most are located 100-150 μm away. The cells have mainly an elongated shape and are of undifferenriated morphology. Under the present conditions, it is estimated that there are at least 360,000 precursor cells in the adult primate brain.

H. Labeled Cells of the Spinal Chord: In this animal, the central canal of some spinal cord regions has been analyzed. While no quantitative analysis has been done, it is clear that, as in mice, BrdU-labeled precursor cells are found scattered along the central canal of the spinal cord (Fig 2).

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I. Summary of Results: In summary, the distribution of BrdU-labeled precursor cells along the rostro-caudal axis of the subventricular zone of the lateral ventricle in the adult primate forebrain closely mimics that found in adult mouse forebrain in that the greatest number of cells is found in the most rostral part of the lateral ventricle. In both mammals, mice and primates, the distribution of the BrdU-labeled precursor cells of the forebrain in the adult correspond to the distribution of the proliferative precursor cells of the germinal matrices associated with the lateral ventricle seen during development, suggesting that this germinal matrices persists into adulthood.

I. Conclusion: In conclusion, this is the first time that the population of proliferating precursor cell of the subventricular zone of the lateral ventricle has been demonstrated in the adult primate forebrain. The precursor cell distribution in adult primate CNS appears to closely mimic that found in mice. During development, the proliferating precursor cells of the subventricular zone migrate out into the forebrain to form neuroglia and neurons. In rodents, the subventricular zone persists into adulthood. Similarly, as described in this study, in primates the subventricular zone persists into adulthood.

5. **Effect of Growth Factors on Primate CNS Precursor Cells**

A. Infusion of Growth Factors:

One female Rhesus monkey (weight 5 kg) was obtained from Connaught laboratory (Mississauga). The growth factors EGF (0.5 μg/kg/h, Chiron) and bFGF (0.2 μg/kg/h, Chiron) were infused directly into the lateral ventricle using an osmotic pump (ALZA, 2ML2; 5μl/h) implanted subcutaneously and connected to a 22 gauge cannula implanted intracerebroventricularly (icv). The vehicle solution was physiological saline containing 0.25 mg/ml rhesus monkey albumin (Sigma A-4287) and 0.065

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mg/ml heparin (Sigma). Heparin is not required for precursor cell preparation, but was used in this study because *in vitro* studies demonstrated that it increases the rate of bFGF-induced precursor cell proliferation. The growth factor mixture was unilaterally infused for 14 continuous days. During the last 5 days of factor infusion, to label CNS precursor cells, the animal was injected intraperitoneally every eight hours for 5 days with BrdU 50 mg/kg (50 mg/mL dissolved in 50% DMSO in 0.09% NaCl; Sigma B-5002).

B. Sacrifice: The animal was killed with an overdose of sodium pentobarbital and perfused through the femoral artery with 2L of physiological saline containing 4000-5000U heparin for 10-20 min. followed by 4L of Bouin's fix for 20-30 min. at 4°C.

C. Tissue Preparation: The brain, spinal cord and a piece of gut (ileum) tissue were dissected out, cut into small blocks and kept in Bouin's postfix solution at 4°C for up to 3 months. The tissue was then transferred to 0.1 M PBS + 0.02% Na-Azide, pH 7.4, at 4°C until ready for cryoprotection. The tissue was cryoprotected by serial incubations at 4°C in 10% sucrose, 20% sucrose and lastly in 20% sucrose:OCT (2:1). The tissue was then embedded in OCT and frozen in an isopentane bath at -45°C and stored at -70°C until sectioning.

D. Cryostat Sectioning: Coronal sections of the rostral part of the lateral ventricle were cut at 30μm from the frontal cortex to the appearance of the third ventricle, with a MICROM cryostat (-20°C) and each section was mounted on gel coated microscope slides and divided into six consecutive series. Slides were stored at -20°C until further processing.

E. BrdU Immunocytochemistry: Slides were washed 3 hr. in PBS (Gibco BRL) to remove the Bouin's fixative and then incubated in 1 M HCl

for 30 min. at 60°C to denature the DNA. This was followed by 3 X 10 min. washes in PBS. The sections were subsequently incubated overnight at room temperature in a primary antibody directed against single-stranded DNA containing BrdU (mouse monoclonal, 1:50, diluted in 0.1 M PBS containing 0.3% triton X-100 and 10% normal serum; Becton). After 3 X 10 min. washes in PBS the slides were incubated at room temperature for 60 min. in goat-anti-mouse CY3 (1:1000; Jackson). Following a final 3 X 10 min. wash in PBS the slides were coverslipped under FluorSave (Calbiochem) and sealed using nail polish. BrdU Immunoreactive cells were then examined using fluorescent microscopy.

F. Quantification of Proliferating Cells: The total number of BrdU-labeled cells surrounding the lateral ventricle was counted using a 40x objective and an ocular grid (250 X 250 μm) which was aligned along the wall of the lateral ventricle or along the contour of the caudate nucleus extending away from the lateral ventricle. The distribution of the cells along the ventricle and along the caudate nucleus was recorded by counting the number of cells within each successive ocular grid placement along the entire dorsal to ventral extent of the lateral ventricle and caudate nucleus. Representative atlas coordinate interaural points were taken as described above in ¶ 4F.

G. Results: Intraventricular infusion of EGF + bFGF + heparin for 14 continuous days into the lateral ventricle of adult primate resulted in a dramatic increase in the total number of BrdU-labeled cells. Attached Figure 2 compares the number of BrdU-labeled precursor cells found ipsilateral to the cannula placement throughout the rostro-caudal axis of the lateral ventricle of the animal that was infused with growth factors compared to the animal which was not infused with growth factors (control animal; see ¶s 4A-

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4J above). The cannula was placed at about the interaural atlas coordinate A20. As can be seen in Figure 2, the number of BrdU-labeled precursor cells in the growth factor treated animal is dramatically increased when compared to the control animal, which received the same BrdU labeling protocol without the growth factor treatment. Furthermore, while BrdU-labeled precursor cells are primarily found within 200 μm of the walls of the lateral ventricle in the control animal, BrdU-labeled precursor cells in the growth factor treated animal are found great distances away from the walls of the lateral ventricle. The maximum migration of labeled cells following the growth factor treatment into the adjacent normal brain parenchyma was approximately 4 mm. Close to the cannulae implantation, proliferating cells are found surrounding cerebral blood vessel suggesting a possible angiogenic effects. The cells induced to proliferate in vivo have a mixture of cell shapes and sizes from elongated small shape or larger amorphous round shape. As observed in adult mice forebrain after in vivo EGF infusion, the increase in the total number of BrdU-labeled cells due to growth factor infusion in the adult primate is also observed on the side contralateral to the cannula placement (Figure 3). In fact, the pattern of cell distribution after growth factor infusion in the adult primate closely mimic the effects of growth factor infusion on BrdU-labeled precursor cells proliferation and migration observed in adult mouse brain.

H. Conclusion: Growth factor infusion into the adult primate brain results in a dramatic increase in the endogenous subependymal neural precursor cell populations and stimulates their migration away from the lateral ventricle walls into adjacent normal brain parenchyma.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that willful, false statements may jeopardize the validity/enforceability of the application or any patent issued thereon.

Dated: May 6/96 Signature: _____
Brent A. Reynolds